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Assay of fungal chitin and estimation of mycorrhizal infection

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In order to estimate the fungal glucosamine resulting from the acid hydrolysis of the chitin contained in the mycorrhizal roots of maritime pines, it is essential to estimate the quantity of amino sugar contained in the tissues of the host plant: this may be estimated by reference to a control sample or by a change in the coloured reaction on the hydrolysate of the mycorrhizal roots. The second method makes it possible to determine the average quantity of these amino sugars contained in the uninfected roots of the pine, and it also makes possible the direct assay of fungal glucosamine without a control sample.

Additional key words — Basidiomycete, glucosamine.

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Résumé. *Pour estimer la glucosamine fongique provenant de l'hydrolyse acide de la chitine contenue dans des mycorhizes de pins maritimes, il est indispensable d'évaluer la quantité de sucres aminés contenue dans les tissus de la plante-hôte : celle-ci peut être évaluée par référence à un échantillon témoin ou par une modification de la réaction colorée sur l'hydrolysat de mycorhizes. Cette deuxième méthode permet d'établir la quantité moyenne de ces sucres aminés contenue dans des racines de Pin non mycorhizées et elle permet en outre de doser directement la glucosamine fongique sans avoir recours à un échantillon témoin.* Mots clés additionnels : basidiomycète, glucosamine.

Abbreviations. DW, dry weight; MBTH, 3-methyl-2-benzothiazolinone hydrazone hydrochloride

INTRODUCTION

Most fungi contain chitin, a polymer of *N*-acetylglucosamine, in their walls (Bartnicki-Garcia, 1968). The assay of this compound indicates a mycelial mass contained in the tissues of a higher plant: this assay has already been used to estimate the mass of pathogen fungi (Ride and Drysdale, 1971; 1972; Toppan *et al.*, 1976; Nicole, 1982), saprophilous fungi (Swift, 1973; Braid and Line, 1981) or endomycorrhizal fungi (Hepper, 1977; Becker and Gerdemann, 1977; Bethlenfalvay *et al.*, 1981; Pacovsky and Bethlenfalvay, 1981).

The same method has been employed for basidiomycete fungi capable of forming ectomycorrhizas: it enabled us to estimate the growth of a mycelium in pure culture (Plassard *et al.*, 1982) and to measure the extent of mycorrhizal infection of maritime pines (Plassard *et al.*, 1983 *a*). In the latter case fungal chitin was assayed by a colorimetric method after alkaline or acid hydrolysis of the plant tissues. Alkaline hydrolysis enables one to assay fungal chitosan specifically; acid hydrolysis, which is much easier to perform is not a specific

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assay for the glucosamine residues from the fungal chitin, and the coloured reaction due to the tissues of the associated phanerogame must also be measured. This requires either the separation of amino sugars from the whole of the residues liberated during hydrolysis (Swift, 1973; Toppan *et al.*, 1976; Braid and Line, 1980), or the use of a control sample of roots without mycelial infection (Plassard *et al.*, 1983 *a* and *b*). This article describes a method which makes it possible to estimate the fungal glucosamine from the acid hydrolysis of maritime pine roots (*Pinus pinaster*) inoculated in controlled conditions or in the nursery, while avoiding the use of a control sample.

MATERIALS AND METHODS

Plant material. The chitin assay was carried out on pure chitin (SIGMA n° C3641), on the thalli of two basidiomycetes (*Hebeloma cylindrosporum* Romagn. and *Pisolithus tinctorius* Coker (Couch.) grown *in vitro* for one month on a Marx modified Melin-Norkrans solution (Marx, 1969), and on uninfected and infected roots of 4 to 5 month old maritime pines (*Pinus pinaster* Soland. *in* Ait.) grown in gnotobiotic conditions on a mixture (v/v) of silicious sand and vermiculite.

Mycorrhizal synthesis. Mycorrhizal synthesis was carried out in growth pouches, according to Fortin *et al.* (1980), and the pines were inoculated with *P. tinctorius* or *H. cylindrosporum*. For the former mycorrhizal fungus, a preliminary culture of the inoculated pines in darkness with high temperature is necessary in order to favour the initial development of mycelium (Plassard *et al.*, 1983 *a*). This is unnecessary for *H. cylindrosporum*. The mycorrhizas were taken out after 3 weeks and each root system mycorrhized with *P. tinctorius* was treated with 3 ml acetone in order to extract the fungal pigments whose assay makes possible the estimation of the mycorrhizal infection (Plassard *et al.*, 1983 *a*). The roots, whether inoculated or not, were dried at 80°C for 24 h. The nursery pines came from the experimental system previously described (Plassard *et al.*, 1983 *b*). They were 30 months old and mycorrhized with natural flora including mainly *Thelephora terrestris*. The preparation of samples was as described previously (Plassard *et al.*, 1983 *b*).

Chitin assay. The samples (a maximum of 100 mg of dry weight or 6.0 mg of pure chitin) were acid-hydrolysed in 5 ml of 6 N HCl, 16 h at 80°C (Swift, 1973). After adjustment of pH of the hydrolysate by the addition of 2.5 ml Na acetate per 0.5 ml of the hydrolysate (Plassard *et al.*, 1982) the glucosamine residues were assayed colorimetrically (Tsuji *et al.*, 1969 *a* and *b*). The colorimetric assay involves two different stages: the glucosamine residues are firstly deaminated by HNO_2, to produce 2-5 anhydromannoses, molecules which possess a free aldehyde group on carbon 1. These compounds then react with MBTH, a specific reaction of aldehyde groups, and give a blue colour in the presence of $FeCl_3$ (Tsuji *et al.*, 1969 *a* and *b*). After acid hydrolysis, it is possible to assay aldehyde groups which do not result from the deamination of amino sugars. The absence of HNO_2 during incubation enables one to determine the quantity of aliphatic aldehydes present in the solution (Sawicki *et al.*, 1961): consequently the quantity of amino sugars may be calculated by the difference between the absorbance values obtained with or without the action of HNO_2.

Colorimetric assay. In order to measure the total absorbance (aldehydes + amino sugars) 1 ml $KHSO_4$ 5% (w/v) and 1 ml $NaNO_2$ 5% (w/v) were added to 1 ml of solution containing glucosamine residues. In order to measure the absorbance due to the aldehyde groups HNO_2 5% was replaced by 1 ml water. After shaking, the mixture was allowed to stand 15 min (deamination time). Then 1 ml $H_2NSO_3NH_4$ 12% was added. After shaking (5 min) 1 ml MBTH 0.5% was added. Reaction time was 1 h without shaking before adding 1 ml $FeCl_3$ 0.5%. The colour was allowed to develop for 30 min; the absorbance was measured at 653 nm, using a sample without glucosamine as blank.

RESULTS AND DISCUSSION

Assay of glucosamine on pure mycelium

The two basidiomycetes grown *in vitro* were subjected to acid hydrolysis. The colour reaction made on the hydrolysate in the presence of HNO_2 results mainly from amino sugars from parietal chitin but also on aldehyde groups which represent 30% and 42% of

Table 1. *Assay of glucosamine on acid hydrolysate from different masses of two basidiomycetes obtained* in vitro. Glucosamine content (with standard error at $p=0.05$) were calculated by method *a* (with the difference in absorbance obtained with or without HNO_2) or method *b* (with absorbance obtained with HNO_2).

Dry weight (mg)	Method	Glucosamine (μg mg^{-1}DW)	
		H. cylindrosporum	*P. tinctorius*
3.0	*a*	75.2± 7.2	56.0±2.6
	b	107.0±22.0	95.0±2.9
6.0	*a*	76.5± 3.7	52.0±3.0
	b	107.5± 4.4	91.1±2.4

the total response respectively for *H. cylindrosporum* and *P. tinctorius (tab. 1)*. On the average chitin supplies 75 μg mg^{-1} dry weight of glucosamine from *H. cylindrosporum* and 54 μg mg^{-1} dry weight from *P. tinctorius*.

Assay of fungal glucosamine

Root-mycelium mixtures samples of 50 and 80 mg of dry matter of uninfected pine roots were mixed with 3 and 6 mg of dry matter of the two fungal types (four replicates of each per couple undergoing treatment) and acid hydrolysed. The estimation of fungal glucosamine in these mixtures was handled in two ways: (*a*) by measuring the absorbance difference (ΔOD) obtained after the colour reactions with or without HNO_2 on aliquot parts of the same hydrolysate (method *a*); (*b*) by measuring the Y' absorbance of an hydrolysate of uninfected pine roots and the Y absorbance of an hydrolysate obtained with a mixture of mycelium and roots (method *b*). The proportion of absorbance due to fungal glucosamine corresponds to the difference ΔY = Y—Y' (Plassard *et al.*, 1983 *a*).

There was good agreement between the values obtained using the two methods: the regression coefficients are close to 1 (*fig.* 1 A and B, curves 1 and 3). The quantities of

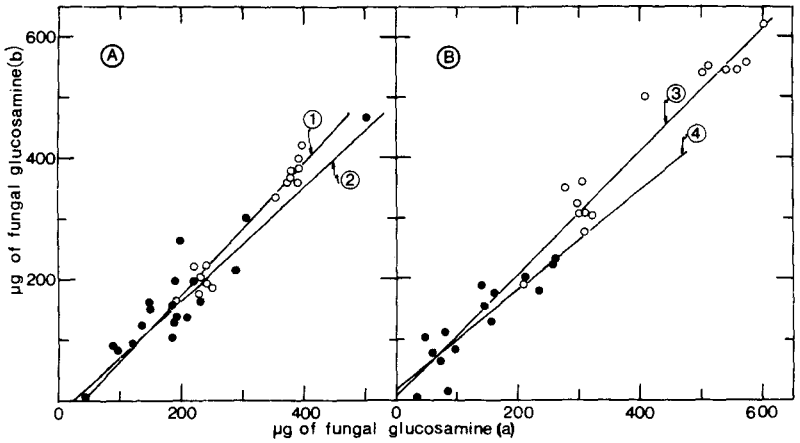

Figure 1. *Acid hydrolysis of two basidiomycetes in the presence of pine roots: relationship between the quantity of fungal glucosamine estimated by methods* a *and* b. **A**, *P. tinctorius*: thalli grown *in vitro* with pine roots (○, curve 1 : $y=1.1x$—48.3; $r=0.984$) or mycelium in the mycorrhizas obtained in pouches (●, curve 2 : $y=0.81x+20.5$; $r=0.948$); **B**, *H. cylindrosporum* : thalli grown *in vitro* with pine roots (○, curve 3 : $y=0.996x+19.25$; $r=0.956$) or mycelium in the mycorrhizas obtained in pouches (●, curve 4 : $y=0.81x+20.5$; $r=0.873$).

Table 2. *Assay of fungal glucosamine on mixtures of uninfected roots and basidiomycetes obtained* in vitro. Glucosamine content (with standard error at $p=0.05$) were calculated by method *a* (with the difference in absorbance (ΔOD) with or without HNO_2 or method *b* (with $\Delta Y = Y—Y'$). (Y' = absorbance obtained with root alone; Y = absorbance obtained with root-mycelium mixtures). Values between brackets were the average glucosamine content for the fungal species and the method employed.

Method	Mycelium (mg DW)	Root (mg DW)	Glucosamine (μg mg^{-1} DW)	
			H. cylindrosporum	*P. tinctorius*
a	3.0	50.0	89.2± 6.0	66.7±6.1
		80.0	85.2±17.0	74.1±6.7
	6.0	50.0	78.1± 8.1	60.0±2.4
		80.0	87.3± 6.1	61.1±0.5
			(85.0±10.0)	(65.4±5.6)
b	3.0	50.0	97.8± 7.4	58.1±8.3
		80.0	83.2±22.0	62.6±3.4
	6.0	50.0	85.2± 3.5	60.0±4.8
		80.0	87.7± 2.6	59.7±1.9
			(88.4±11.7)	(60.0±1.6)

fungal glucosamine measured on the hydrolysate of a mixture of roots and mycelium were not significantly different whatever the method employed. This should not be the case, as in the first method (method *a*) absorbance is only due to amino sugars whereas in the second method (method *b*) it results from all the aldehyde groups. In addition, the comparison of tables 1 and 2 indicates that the quantities of glucosamine assayed in the mycelium are different when the assay is carried out on the mycelium alone or on a mixture with roots.

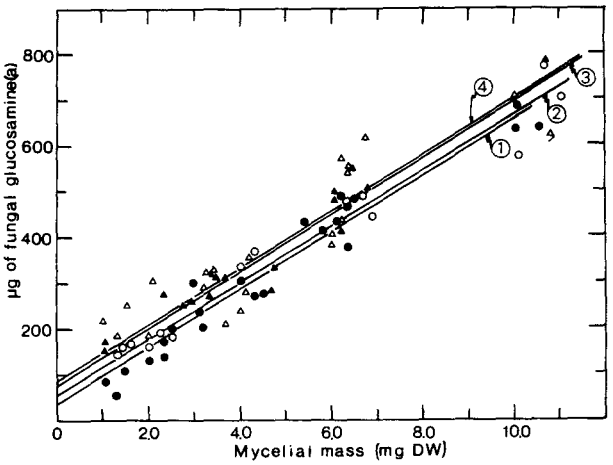

Figure 2. *Quantity of fungal glucosamine estimated by method* a *in relation to the mass of dry matter of* H. cylindrosporum *hydrolysed alone or in presence of pine roots.* (●), curve 1, mycelium alone : $y=61.6x+37.8$; $r=0.967$; (○), curve 2, mycelium + 20 mg DW of pine roots: $y=60.3x+56.4$; $r=0.978$; (▲), curve 3, mycelium + 50 mg DW of pine roots: $y=61.5x+80.8$; $r=0.978$; (△), curve 4, mycelium + 80 mg DW of pine roots: $y=60.1x+88.4$; $r=0.943$. In curves 3 and 2, the Y-intercepts are significantly different ($p=0.05$) from that obtained with mycelium alone (curve 1).

Method *a* enables one to measure absorbance due specifically to amino sugars. The quantity of glucosamine thus determined in a hydrolysate of pine roots is 1.02 ± 0.55 µg mg^{-1} dry weight. When increasing quantities of mycelium of *H. cylindrosporum* mixed with different quantities dry root matter (20, 50 and 80 mg), the quantities of glucosamine increase proportionately to the mass of mycelial tissues (*fig.* 2). Absorbance due to the amino sugars of roots is added to that due to the glucosamine of the mycelium, as is indicated by Y-intercepts which are different for the curves of figure 2. In these conditions more glucosamine is found than in the mycelium alone (*tab.* 1 and 2).

Method *b* depends on one assumption: the absorbance values measured for samples resulting from the hydrolysis of a mixture of roots and mycelium represent the total absorbance values given by these two tissues (Plassard *et al.*, 1983 *a*). In fact the values measured by method *b* on mixtures of two plants (*tab.* 2) were all systematically lower than those measured on the mycelium alone (*tab.* 1): this is in contradiction with the initial assumption. The presence of pine roots would therefore seem to slightly decrease the absorbance measured after the colour reaction although this interaction is difficult to establish in a significant manner by means of statistical calculation (Plassard *et al.*, 1983 *a*).

In addition the proportion of absorbance due to the roots is higher, by one order of magnitude, than that due to the mycelium as the masses of the two plants in the samples are very different. Consequently the variability of the assays of the control sample of roots contributes heavily to the random error in the determination of the real quantity of glucosamine.

Assay of fungal glucosamine on mycorrhizal pines

Roots of maritime pines, inoculated either experimentally with *H. cylindrosporum* and *P. tinctorius* or by natural flora in the nursery, were subjected to acid hydrolysis. Fungal glucosamine was estimated by methods *a* and *b*. In the case of pines from the nursery, the

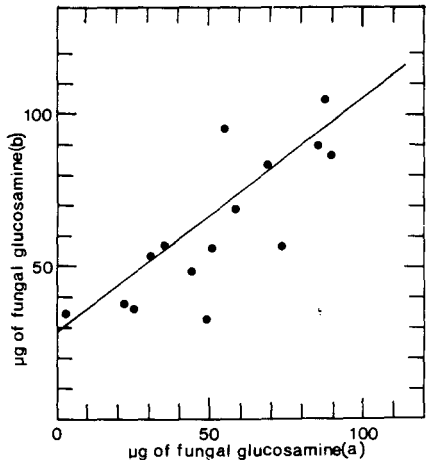

Fig. 3

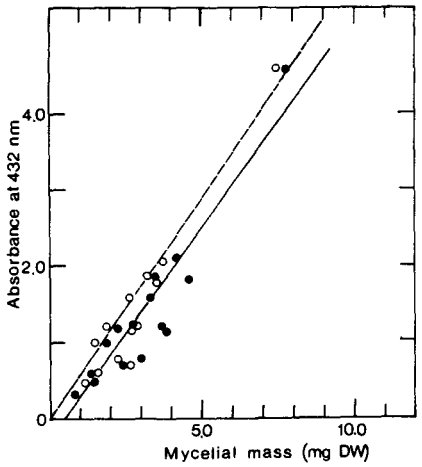

Fig. 4

Figure 3. *Relationship between the quantity of fungal glucosamine estimated by methods* a *and* b *after acid hydrolysis lateral roots from nursery pines,* $y = 0.76x + 23.3$; $r = 0.78$.

Figure 4. *Relationship between the quantity of root pigments infected by* P. tinctorius *given by the measure of absorbance at* 432 *nm, and the mycelial mass estimated by methods* a *and* b. (●), solid line, method *a*: $y = 0.575x - 0.29$; $r = 0.916$; (○), dotted line, method *b*: $y = 0.581x - 0.03$; $r = 0.910$.

control samples of method *b* were the tap roots of the root system (Plassard *et al.*, 1983 *b*). Figure 1 A and B (curves 2 and 4) indicates that there still exists a good concordance (regression coefficient close to 1) between the two estimations of the quantities of fungal glucosamine of the mycorrhizas obtained in pouches. The same is true for the pines grown in the nursery (*fig.* 3). The curves obtained with mycorrhizas of *H. cylindrosporum* or of *P. tinctorius* do not differ in any significant manner from those obtained by artificially mixing mycelium and roots grown separately (*fig.* 1 A and B).

The degree of mycorrhizal infection of pines inoculated with *P. tinctorius* was estimated by measuring the absorbance at 432 nm of an acetone extract of the pigments of the fungus (Plassard *et al.*, 1983 *a*). At the same time the mycelial masses in these mycorrhizas were estimated by calculating the ratio of the total quantity of glucosamine (method *a* or *b*) and the average quantity of glucosamine contained in the mycelium of *P. tinctorius* grown *in vitro* and hydrolysed with pine roots (62.7 $\mu g\ mg^{-1}$ dry weight, table 2). The correlations between absorbance at 432 nm and the mycelial mass thus estimated are highly significant and the curves obtained are not significantly different regardless of the method employed (*fig.* 4).

CONCLUSION

The choice of acid hydrolysis for the assay of fungal chitin of isolated mycelium or mycorrhizas is justified by the facility of operation as opposed to alkaline hydrolysis which requires numerous manipulations (Braid and Line, 1981). On the other hand the method of assay of the glucosamine residues thus obtained is not strictly specific. The aldehyde groups are sensitive to the coloured reaction. The fact that this reaction takes place before the deamination of the glucosamine by HNO_2 enables one to subtract the absorbance due to these aldehyde groups from that due specifically to glucosamine. Nevertheless the error introduced by the amino sugars from the tissues of the host plant is not eliminated. The results obtained indicate that these amino sugars are equivalent to 1.02 ± 0.55 µg of glucosamine per mg of dry weight of root (mycorrhiza or host tissue) beyond this value fungal chitin is detected. The method we propose avoids the problems posed by reference to a control sample. The good correlation with the other methods usually employed (Plassard *et al.*, 1983 *a*) suggests that the assay of chitin by the method of Tsuji *et al.* (1969 *b*), after the acid hydrolysis of the plant material, constitutes a good means of estimating the intensity of mycorrhizal infection.

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